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Could Mannitol-Induced Delay of Anoxic Depolarization be Relevant in Stroke Patients?

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1. Introduction

The use of hyperosmotic agents in stroke is still a matter of debate, since their usefulness has repeatedly been suggested but not conclusively demonstrated (Righetti E et al., 2002). Better understanding of the possible mechanism of protection by hyperosmotic agents may help identifying clinical situations where they may be more useful. It is generally assumed that their effect in stroke is due to their capacity to reduce brain edema. However, increasing extracellular osmolarity has direct effects on neuronal electrical function (Osehobo and Andrew, 1993; Rudehill et al., 1993), and one of us has previously reported that adding mannitol to the perfusing medium of brain slices delays anoxic depolarization (AD) (Balestrino, 1995a; Balestrino, 1995b). Since the latter is a factor in causing neuronal damage in anoxia and ischemia (Balestrino and Somjen, 1986; Jarvis et al., 2001; Kaminogo et al., 1998; Somjen et al., 1990), this may be another mechanism of brain protection by hyperosmotic agents in stroke. This study investigates whether or not this delay occurs at values of hyperosmolarity that may be obtained in clinical practice. We first carried out a survey of the literature on osmolarity changes after administration of hyperosmotic agents in vivo, under both clinical and experimental conditions. Then, we did a dose-response study of mannitol-induced delay of AD. Finally we compared the two sets of data to gauge whether or not mannitol-induced delay of AD occurs in the range of hyperosmolarity that might be obtained in clinical practice.

2. Materials and methods

Sprague-Dawley female rats (155-190g.) were anaesthetised with ether and decapitated. The left hippocampus was dissected free and cut in 600 μ m thick transversal slices. Slices were immediately transferred into an "interface" recording chamber (Fine Science Tools, Vancouver B. C. Canada) and incubated at $35\pm 1^\circ\text{C}$. They were bathed by Artificial CerebroSpinal Fluid (ACSF) flowing at 2 ml/min and having the following composition: NaCl 130 mM, KCl 3.5 mM, NaH_2PO_4 1.25 mM, NaHCO_3 24 mM, CaCl_2 2.4 mM, MgSO_4 1.2 mM, glucose 10 mM. This medium was continuously bubbled with 95% O_2 / 5% CO_2 , resulting in a pH of 7.35-7.40. The same warmed, humidified 95% O_2 / 5% CO_2 mixture aerated the slices representing the gas phase. Anoxia was induced by replacing oxygen with

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nitrogen in the gas phase. The DC-coupled, ground-referenced extracellular potential of the tissue was constantly monitored in the cell body layer of CA1. As soon as the sudden fall in this potential that is the hallmark of AD was observed, oxygen flow was restored. A cross-over study design was observed, with the same slice being subjected to anoxia, at 30' intervals, both in the presence and in the absence of mannitol. Each slice was subjected to two anoxic episodes. The sequence of treatments (mannitol first, or control ACSF first) was alternated in consecutive experiments, to minimize the bias due to possible effects of repeated anoxia *per se* on AD latency. In two experiments, the same slice was subjected to three anoxic episodes the first one in mannitol, the second in control ACSF, the third one in mannitol again. For statistical analysis, in each experiment the difference in latency between AD in mannitol and AD in control ACSF was computed, and used as a gauge of mannitol efficacy in that experiment.

3. Results

3.1 Literature search on serum osmolarity changes in vivo

Results are summarized in figure 1 and in Table 1.

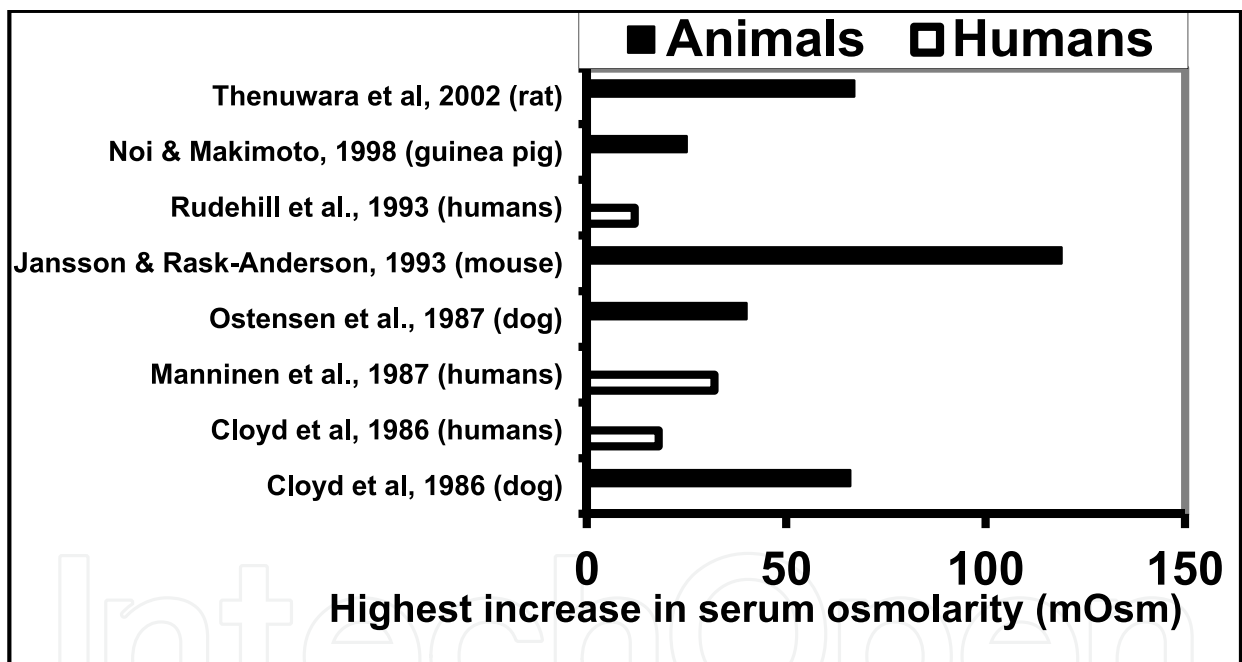


Fig. 1. **Increases in serum osmolarity reported in the literature:** This figure depicts the highest increase in serum osmolarity reported in each of the papers listed in Table 1. It refers to papers quoted in the Reference List. This figure is meant to graphically visualize the highest reported increases. Refer to Table I and to text for further information.

In human patients, use of mannitol at the dose of 0.5-2 g/Kg body weight is reported (Cloyd et al., 1986; Newman, 1979; Rudehill et al., 1993). Such a dose leads, still in human patients, to a maximum increase in serum osmolarity of about 10-32 mOsm (Cloyd et al., 1986; Manninen et al., 1987; Rudehill et al., 1993). When experimental animals are considered, administration of 1 g/Kg body weight to rats yielded a serum osmolarity increase of 4 mOsm (Thenuwara et al., 2002). In dogs, mannitol administration of 0.5, 1 or 1.5 g/Kg lead to a peak increase (mean±SD) of 43±18, 66±18 and 52±23 mOsm, respectively, during the brief time of the infusion, and to the

Paper	Animal species	Osmotic agent infused	Dose	Serum osmolarity increase
(Cloyd et al., 1986)	Humans	Mannitol	0.5, 0.7 g/Kg	10 - 18 mOsm
(Cloyd et al., 1986)	Dog	Mannitol	0.5, 1, 1.5 g/Kg	43-66 mOsm
(Jansson and Rask-Anderson, 1993)	Mice	Glycerol	1.3, 2.6 and 5.2 g /kg	12-119 mOsm
(Manninen et al., 1987)	Humans	Mannitol	1, 2 g/Kg	32 mOsm
(Newman, 1979)	Humans	Mannitol	2 g/Kg	Not reported
(Noi and Makimoto, 1998)	Guinea pig	Glycerol	30-min infusion of 50% glycerol	6 mOsm
(Noi and Makimoto, 1998)	Guinea pig	Urographin®	30-min infusion of 76% Urographin®	25 mOsm
(Ostensen et al., 1987)	Dog	Mannitol		40 mOsm
(Rudehill et al., 1993)	Humans	Mannitol	1 g/Kg	12 mOsm.
(Thenuwara et al., 2002)	Rat	Mannitol	1, 4, 8 g/Kg, with or without furosemide	4-67 mOsm

Table 1. Literature data on changes in serum osmolarity after i.v. infusion of osmotic agents. The table summarizes available literature data on changes in serum osmolarity after i.v. infusion of osmotic agents. When different changes in osmolarity are reported following different doses of osmotic agent, in the table the range of increases is given. The values given in the table are either the numbers provided by the Authors or those obtained by measuring graphs in their papers. In the latter case, the value is obviously less precise. When the Authors reported mean±SD for pre- and post-infusion osmolarity, in the table the corresponding difference between means is given.

lower increase of 10 mOsm or less afterwards (Rudehill et al., 1993). Higher doses of mannitol (4 g/Kg and 8 g/Kg, with or without the addition of furosemide) lead, in rats, to a rather high serum osmolarity increase, reaching an average as high as 67 mOsm (Thenuwara et al., 2002). Under experimental conditions in vivo, glycerol infusion leads to average increases in serum osmolarity of 6 mOsm in guinea pigs (Noi and Makimoto, 1998) and of up to 119 mOsm in dogs (Jansson and Rask-Anderson, 1993). Using Urografin® infusion, a 25 mOsm increase in serum osmolarity was obtained in guinea pigs (Noi and Makimoto, 1998).

3.2 Effects of increasing mannitol in vitro

The previously shown robust effect of mannitol in delaying AD was first confirmed in double wash-out experiments, where the same slice was subjected to transient anoxia in the presence of mannitol, then in control ACSF, then again in the presence of mannitol. These experiments are illustrated in figure 2. Mannitol concentrations of 100 and 500 mM were used in these experiments, as they were those that had been previously shown to most reliably delay AD (Balestrino, 1995). The effects of mannitol were then investigated at different concentrations. Fig. 3 summarizes these results. As it can be seen, 1 and 10 mM were not effective in delaying AD. Twenty-five mM significantly delayed AD, while 50 mM did not show a statistically

significant effect. The quite high concentrations of 100 and 500 mM significantly increased the latency of AD.

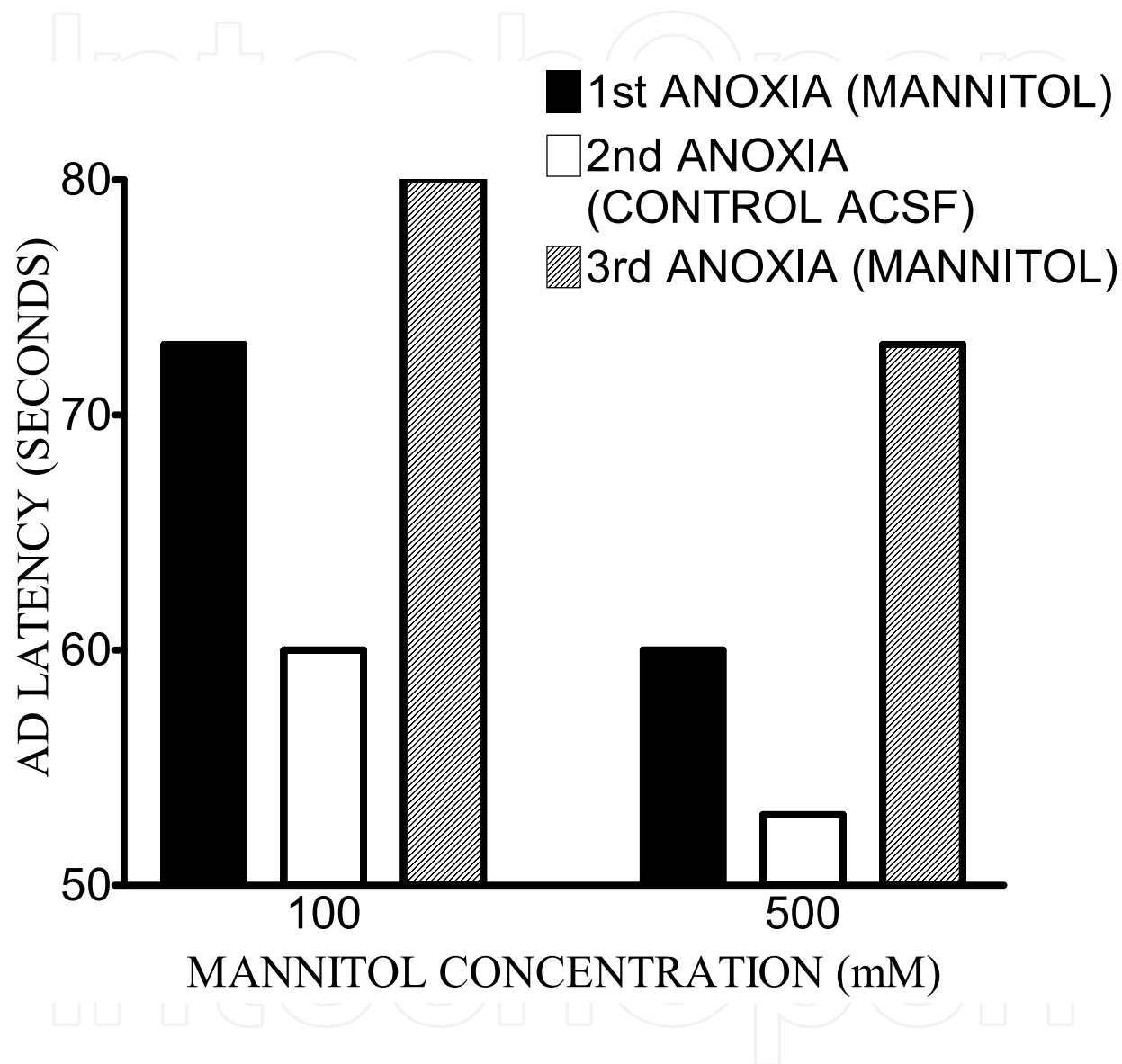


Fig. 2. **Double wash-out experiments showing mannitol effectiveness in delaying anoxic depolarization.** In two different slices, anoxia was induced in mannitol-fortified Artificial CerebroSpinal Fluid (ACSF), then in control ACSF, then again in ACSF with added mannitol. In one experiment (set of bars at left) 100 mM mannitol were used, in the other (set of bars at right) 500 mM mannitol were used. Bars represent latency of AD in each anoxia episode. Control ACSF reversibly decreased AD latency, thus confirming the previously published efficacy of mannitol in increasing AD latency.

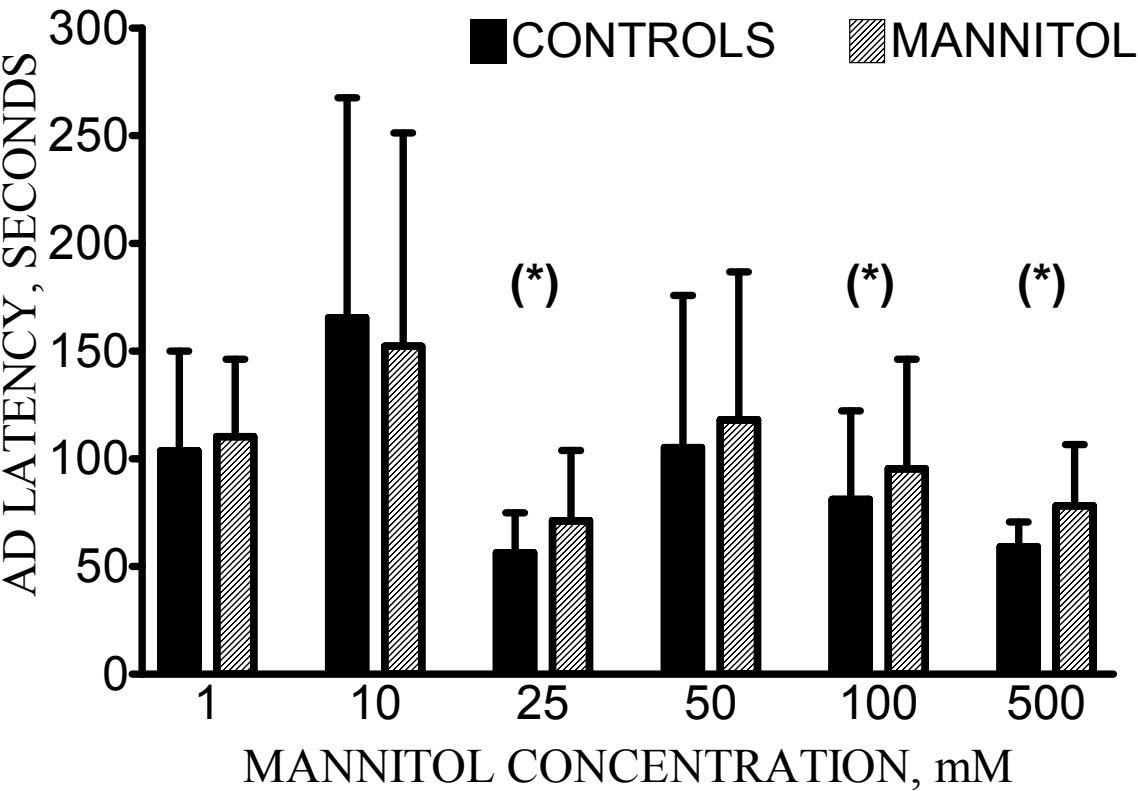


Fig. 3. Effects of different mannitol concentrations in delaying anoxic depolarization. The bars show latency of AD (mean± SD)in both control and mannitol-fortified ACSF for different mannitol concentrations. Asterisks mark the groups in which the difference is statistically significant ($p<0.03$, t-test for paired data). N=3 for 1mM, N=4 for 10 mM, N=8 for 25 mM, N=7 for 50 mM, N=6 for 100 mM, N=6 for 500 mM. See text for experiment design.

Figure 4 shows an example of AD delay by mannitol. In a further analysis, we calculated for each slice the difference between the latency of AD in mannitol and the latency of AD in control ACSF. Such a difference was used as a gauge of mannitol effectiveness in that particular slice. If the difference had been positive, it would have indicated that latency in mannitol was longer than in control (i.e., AD occurred later), thus showing protection by mannitol and quantifying its degree. The opposite would have been true for a negative difference. Results are shown in Figure 5. As it can be seen, above 10 mM all concentrations of mannitol delay AD to a comparable extent. Such a finding has already been reported, from our laboratory, for AD delay by creatine (Balestrino, 1995).

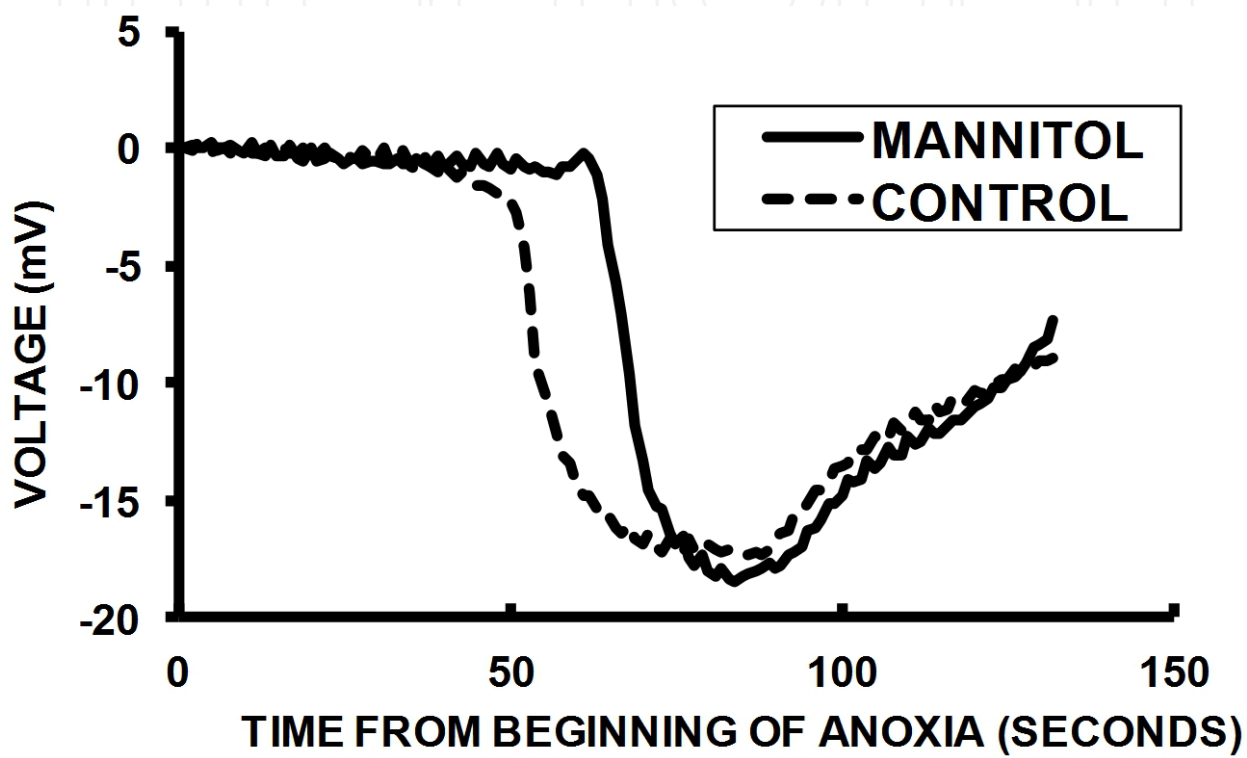


Fig. 4. **Sample anoxic depolarization in control and mannitol-treated ACSF.** Two different anoxic episodes in the same slice. The dotted line represents DC tracing (showing anoxic depolarization) during anoxia in control ACSF, the solid line represents the same tracing during anoxia in ACSF with added mannitol. AD occurs later in mannitol-fortified ACSF.

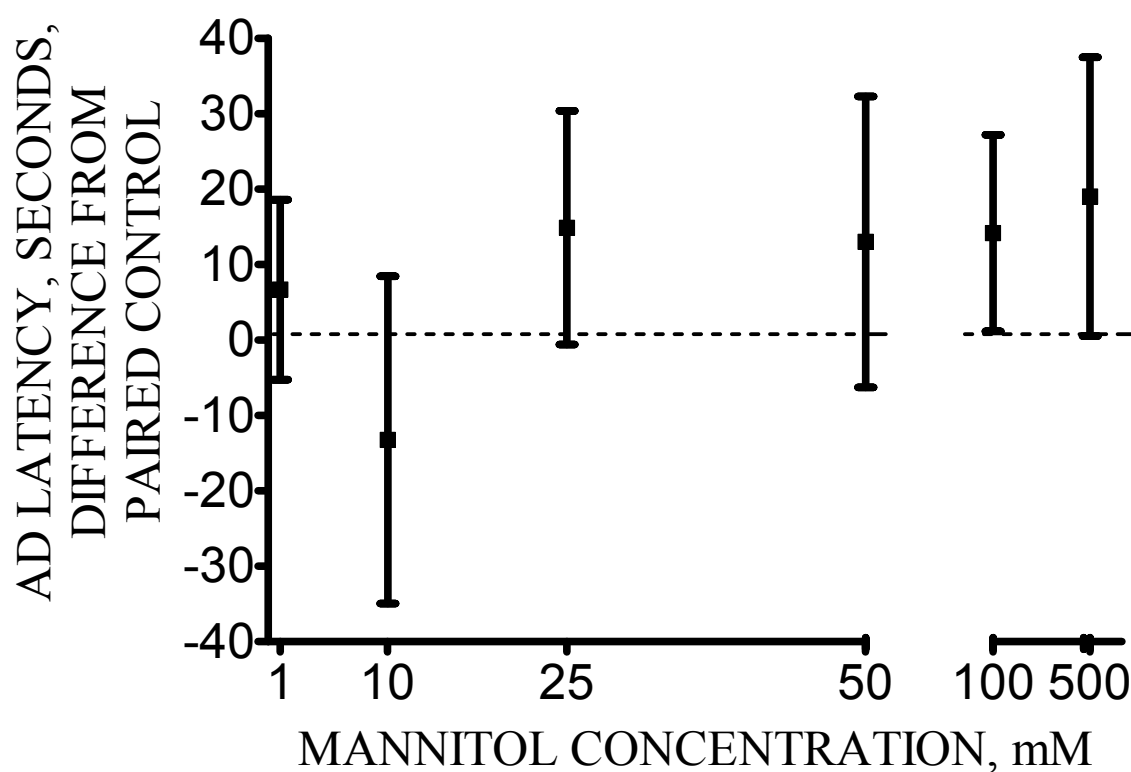


Fig. 5. **Measure of AD delay in different mannitol concentrations.** Same experiments as in figure 3. This figure depicts more precisely the increase in AD latency determined by mannitol at each concentration. For each slice, the difference (AD latency in ACSF with mannitol) – (AD latency in control ACSF) was computed. A positive difference means that AD latency was longer in mannitol (i.e., AD occurred later), the opposite is true for a negative difference. Data were grouped for mannitol concentration. For each concentration, mean±SD is provided. Number of experiments as in figure 3. Concentrations of 25 mM mannitol and higher all delay AD to the same extent.

4. Discussion

The effectiveness of hyperosmolarity in delaying AD was confirmed by these findings. Delay of AD may be relevant to neuroprotection in stroke, because AD is a factor in the generation of anoxic damage, and its delay has been associated with better outcome under experimental conditions (Balestrino and Somjen, 1986; Jarvis et al., 2001; Kaminogo et al., 1998; Somjen et al., 1990). The present study indicates that significant delay of AD is obtained at mannitol concentrations greater than 10 mM, 25 mM being the lowest effective dose among those tested. An overview of the literature showed that in human patients serum osmolarity increases, under common clinical settings, by 10-32 mOsm after administration of 1 g/Kg mannitol (Table 1 and fig. 1). This is equivalent to adding 10-32 mM mannitol to in vitro slices¹. At the lower end of this range, such an

¹ Since the molecule of mannitol does not split in aqueous solutions, the molarity of mannitol in solution (here expressed in mM) corresponds to the consequent increase in osmolarity (1mM=1mOsm).

increase would be insufficient (10 mM (Cloyd et al., 1986; Rudehill et al., 1993)) or perhaps barely sufficient (18 mM (Cloyd et al., 1986)) to afford delay of AD. In the upper end, a 32 mM increase (Manninen et al., 1987) would probably be somehow effective in delaying AD. In fact, we showed that in vitro the addition of 25 mM mannitol (increasing osmolarity by 25 mOsm) significantly delays AD. The delay in AD was not significant after addition of 50 mM (increasing osmolarity by 50 mOsm), indicating that these osmolarity increases (25-50 mOsm) are of borderline efficacy. However, much higher increases, up to 100 mOsm and more, were reliably effective in vitro, and have been reported under experimental conditions in laboratory animals, apparently without severe adverse effects (Table 1 and figure 1). The latter increases would be in a range that does cause AD delay (compare figure 5 with figure 1). If further studies suggested that a comparable increase in serum osmolarity can be safely obtained in human patients, it might be useful not only by decreasing brain edema, but also by having a direct effect on tissue depolarization.

Two more considerations are in order.

First, in human stroke mannitol or other hyperosmotic agents would be administered when AD has already occurred. In fact, AD is an event that occurs in the core of an infarction soon after ischemia. Nevertheless, under experimental conditions continuous or repeated depolarizations have been demonstrated in the hours following stroke (Chen et al., 1993; Ohta et al., 1997). Their reduction has been associated with better outcome (Chen et al., 1993). Given the striking similarity of these events with “classical” AD, it is very likely that hyperosmolarity can delay or suppress these waveforms as well, thus providing protection.

Second, the changes reported in the literature are in serum, not in the interstitial space of the brain. To the best of our knowledge, no study has yet measured increases in osmolarity in the interstitial space of the brain, probably due to the technical difficulty of this investigation. However, it is reasonable to assume that an increase in osmotic pressure in the serum draws water from the brain interstitial space, thus increasing its osmolarity to a comparable degree. Therefore, increase in serum osmolarity should be comparable, at least to an extent, to increase in osmolarity of the brain interstitial space.

Finally, it should be noted that future clinical studies on hyperosmotic agents in stroke should take into account the increase in serum osmolarity that was obtained in the single patients. In fact, our data indicate that the latter is a critical variable in determining whether the hyperosmotic therapy will be effective or not.

Summing up, we conclude that the increase in serum osmolarity that is commonly obtained in clinical practice is not sufficient to delay AD. Larger increases in serum osmolarity have been, however, reported in animal experiments. If further studies indicated that such increases were safe in humans as well as in animals, they might provide brain protection by decreasing AD and AD-like depolarizations. Future clinical studies on hyperosmotic agents in stroke should measure and take into account the degree of changes that were obtained in serum osmolarity.

5. Acknowledgment

We thank Prof. Aroldo Cupello for his useful comments on the manuscript.

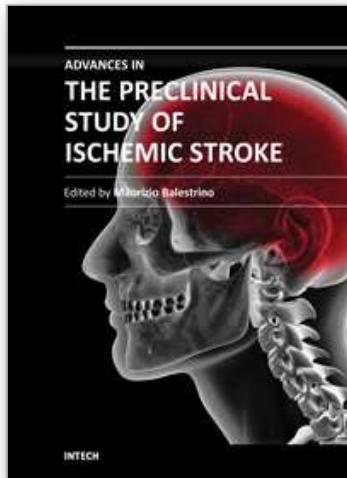
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Advances in the Preclinical Study of Ischemic Stroke

Edited by Dr. Maurizio Balestrino

ISBN 978-953-51-0290-8

Hard cover, 530 pages

Publisher InTech

Published online 16, March, 2012

Published in print edition March, 2012

This book reports innovations in the preclinical study of stroke, including - novel tools and findings in animal models of stroke, - novel biochemical mechanisms through which ischemic damage may be both generated and limited, - novel pathways to neuroprotection. Although hypothermia has been so far the sole "neuroprotection" treatment that has survived the translation from preclinical to clinical studies, progress in both preclinical studies and in the design of clinical trials will hopefully provide more and better treatments for ischemic stroke. This book aims at providing the preclinical scientist with innovative knowledge and tools to investigate novel mechanisms of, and treatments for, ischemic brain damage.

How to reference

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Maurizio Balestrino, Enrico Adriano and Patrizia Garbati (2012). Could Mannitol-Induced Delay of Anoxic Depolarization be Relevant in Stroke Patients?, *Advances in the Preclinical Study of Ischemic Stroke*, Dr. Maurizio Balestrino (Ed.), ISBN: 978-953-51-0290-8, InTech, Available from: <http://www.intechopen.com/books/advances-in-the-preclinical-study-of-ischemic-stroke/could-mannitol-induced-delay-of-anoxic-depolarization-be-relevant-in-stroke-patients->

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